

Identification of Common and Distinct Residues Involved in the Interaction of α_{i2} and α_s with Adenylyl Cyclase*

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The G protein α subunits, α_s and α_{i2} , have stimulatory and inhibitory effects, respectively, on a common effector protein, adenylyl cyclase. These effects require a GTP-dependent conformational change that involves three α subunit regions (Switches I–III). α_s residues in three adjacent loops, including Switch II, specify activation of adenylyl cyclase. The adenylyl cyclase-specifying region of α_{i2} is located within a 78-residue segment that includes two of these loops but none of the conformational switch regions. We have used an alanine-scanning mutagenesis approach within Switches I–III and the 78-residue segment of α_{i2} to identify residues required for inhibition of adenylyl cyclase. We found a cluster of conserved residues in Switch II in which substitutions cause major losses in the abilities of both α_{i2} and α_s to modulate adenylyl cyclase activity but do not affect α subunit expression or the GTP-induced conformational change. We also found two regions within the 78-residue segment of α_{i2} in which substitutions reduce the ability of α_{i2} to inhibit adenylyl cyclase, one of which corresponds to an effector-activating region of α_s . Thus, both α_{i2} and α_s interact with adenylyl cyclase using: 1) conserved Switch II residues that communicate the conformational state of the α subunit and 2) divergent residues that specify particular effectors and the nature of their modulation.

Upon activation by cell surface receptors, heterotrimeric G proteins transmit signals to effector proteins that regulate a wide variety of cellular processes (1–4). Receptors activate G proteins by catalyzing the replacement of GDP bound to the α subunit with GTP, resulting in dissociation of α -GTP from the $\beta\gamma$ subunits. The GTPase activity of the α subunit regulates the timing of deactivation and reassociation of the G protein subunits. The fidelity of cellular signaling requires that α subunits modulate effector proteins only when bound to GTP and that only the appropriate α subunit-effector pairs interact. GTP-dependent effector interaction most likely involves one or more of the three α subunit regions that change conformation during the GTPase cycle (Switches I–III), identified by comparison of the x-ray crystal structures of the GTP γ S-bound¹ (active) and GDP-bound (inactive) forms of α_t (5, 6) and α_{i1} (7, 8). Differ-

ences in the amino acid sequences of the structurally conserved α subunits (40% identity at the amino acid level, with 60–90% identity within subfamilies) determine the specificity and nature of their interactions with effector proteins (9). However, the relationship between the molecular determinants of effector specificity and of GTP-dependent effector regulation is poorly understood.

Regulation of adenylyl cyclase by the G protein α subunits, α_s and α_i , raises issues specific for this α subunit-effector interaction. α_s and α_i , which are relatively poorly conserved among the family of α subunits (~40% identical amino acids), both bind to adenylyl cyclase but have opposite effects on activity. Inhibition of adenylyl cyclase by α_i requires prior activation by α_s , forskolin, or calmodulin (10, 11). Since adenylyl cyclase can be inhibited by α_i in the absence of α_s , inhibition does not appear to be due to competition between α_i and α_s for binding to adenylyl cyclase. Indeed, there is evidence that suggests that adenylyl cyclase has distinct binding sites for α_s and α_i (11). Key questions that arise are: why does α_s activate and α_i inhibit, and why do only α_s and α_i , but not other α subunits, modulate adenylyl cyclase activity?

The α_s residues that specify activation of adenylyl cyclase are located in three adjacent loops, one of which includes Switch II (12). The location of a conformational switch region within the effector-specifying surface of α_s provides a simple mechanism for the GTP-dependence of the α_s -adenylyl cyclase interaction. However, studies with chimeric α subunits containing portions of α_{i2} and α_q , which does not interact with adenylyl cyclase (13), showed that an $\alpha_q/\alpha_{i2}/\alpha_q$ chimera containing only 78 residues of α_{i2} (residues 245–322) inhibits adenylyl cyclase as well as α_{i2} does (14). This 78-residue effector-specifying segment includes residues homologous to two of the three clusters of α_s residues that specify activation of adenylyl cyclase (12, 15) but does not include any of the conformational switch regions. This was a surprise since the GTP-bound form of α_i is much more effective at inhibiting adenylyl cyclase than the GDP-bound form is (11). However, the importance of the conformational switch regions might have been missed using a chimeric α subunit approach due to the high degree of sequence similarity in these regions between α_q and α_{i2} .

To determine whether any of the conformational switch regions are involved in inhibition of adenylyl cyclase by α_{i2} , we substituted alanines for solvent-exposed residues in these regions. We tested the effect of these mutations on both the inhibition of adenylyl cyclase and the ability of the mutant proteins to achieve the activated conformation as measured by the acquisition of trypsin resistance upon binding of GTP. We identified a part of Switch II that is conserved among α subunits in which alanine substitutions blocked the inhibition of adenylyl cyclase by α_{i2} . We also found that substitutions of alanines for the corresponding α_s residues specifically prevent activation of adenylyl cyclase. Thus it appears that both α_{i2} and

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¹ The abbreviations used are: GTP γ S, guanosine 5'-O-(thiotriphosphate); hGH, human growth hormone; PDE, cGMP phosphodiesterase; PLC, phosphoinositide phospholipase C.

α_s interact with adenylyl cyclase using two types of residues: 1) conserved residues within Switch II that signal that the α subunit is in the GTP-bound active conformation and 2) divergent residues that specify activation or inhibition of this effector enzyme.

To identify the α_{i2} residues involved in specifying inhibition of adenylyl cyclase, we substituted alanines for solvent-exposed residues within the 78-residue segment. We found two regions of sequence in which mutations impaired the ability of α_{i2} to inhibit adenylyl cyclase, the amino terminus of α_3 and the α_4/β_6 loop. The α_4/β_6 loop is also important for the effector interactions of α_s (12) and α_t (16, 17). These substitutions did not cause as much of a decrease in adenylyl cyclase inhibition as the Switch II mutations did, suggesting that Switch II residues are the primary contributors to the interaction between α_{i2} and adenylyl cyclase.

EXPERIMENTAL PROCEDURES

Generation of Plasmids— α_{i2} mutants were constructed from the mouse α_{i2} cDNA (18), and α_s mutants were constructed from the rat α_s cDNA (19). Two modifications were made to each of the α subunits to facilitate detection of their activities and expression levels. The arginine at position 179 in α_{i2} and 201 in α_s was mutated to cysteine to inhibit GTPase activity and produce constitutive activation (20, 21). An epitope, referred to as the EE epitope (22) was generated by mutating α_{i2} residues SDYIPTQ (166–172) to EEYMPTE and α_s residues DYVPSD (189–194) to EYMPTE (single letter amino acid code, mutated residues are underlined). The resultant constructs were designated α_{i2} RCEE and α_s RCEE respectively. α_s RCEE was generated from the rat α_s cDNA (19) by mutating arginine 179 to cysteine and residues DYQPT (167–172) to EYMPTE.

The α_{i2} RCEE cDNA (gift of Ann Pace and Henry Bourne, University of California, San Francisco) was subcloned into pcDNA I/Amp (Invitrogen) as an *EcoRI* fragment. The α_s EE cDNA (gift of Paul Wilson and Henry Bourne, University of California, San Francisco) was subcloned into pcDNA I/Amp as a *HindIII* fragment. To produce the α_s RC cDNA, the α_s RCHA cDNA (12), which contains the HA epitope from influenza virus (23), was digested with *XbaI* and *EcoRI* to yield a fragment containing the R201C mutation but not the HA epitope. *XbaI-EcoRI* restriction of α_s EEpcDNA I/Amp removed a fragment containing the EE epitope, which was replaced by the *XbaI-EcoRI* fragment from the α_s RCHA cDNA to produce α_s RCpcDNA I/Amp. To generate α_s RCEEpcDNA I/Amp, α_s RCpcDNA I/Amp was digested with *AlwI* to yield a fragment containing the R201C mutation, which was ligated into α_s EEpcDNA I/Amp in place of the analogous fragment to produce an α_s cDNA containing both the R201C mutation and the EE epitope.

All mutations were generated by oligonucleotide-directed *in vitro* mutagenesis (24) using the Bio-Rad Muta-Gene kit except for those in the α_{i2} RCEE derivatives, Constructs 2 and 3, which were produced by polymerase chain reactions that generated DNA fragments with overlapping ends that were subsequently combined in a fusion polymerase chain reaction (25). All mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing.

cAMP Accumulation Assay—Recombinant α subunits were transiently expressed in the human embryonic kidney fibroblast line, HEK-293 (American Type Culture Collection CRL-1573), using DEAE-dextran (26) under the control of the cytomegalovirus promoter in the expression vector, pcDNA pcDNA I/Amp. To measure inhibition of adenylyl cyclase, 10^6 cells/60-mm dish were co-transfected with 0.1 μ g of vector containing α_s RC and 0.3 μ g of vector containing α_{i2} RCEE, α_s RCEE, or mutant derivatives of α_{i2} RCEE. To measure activation of adenylyl cyclase, 10^6 cells/60-mm dish were transfected with 1.5 μ g of vector containing α_s RCEE or mutant derivatives of this construct or with vector alone. Intracellular cAMP levels in cells labeled with [3 H]adenine were determined as described (14).

Membrane Preparations and Trypsin Assay—HEK-293 cells were transiently transfected with recombinant α subunit constructs using DEAE dextran (26). Membranes were prepared 48 h after transfection as described (14). For the trypsin resistance assay (12), membrane proteins (70 μ g) were diluted to a concentration of 6 mg/ml in a buffer containing 20 mM HEPES (pH 8.0), 10 mM MgCl₂, 1 mM EDTA, 2 mM β -mercaptoethanol, and 0.64% (w/v) of the detergent lubrol PX. Solubilized proteins were collected after centrifugation for 10 min at 4 °C in a microcentrifuge and incubated for 30 min at 30 °C in the presence or absence of 125 μ M GTP γ S. Tosylphenylalanyl chloromethyl ketone-

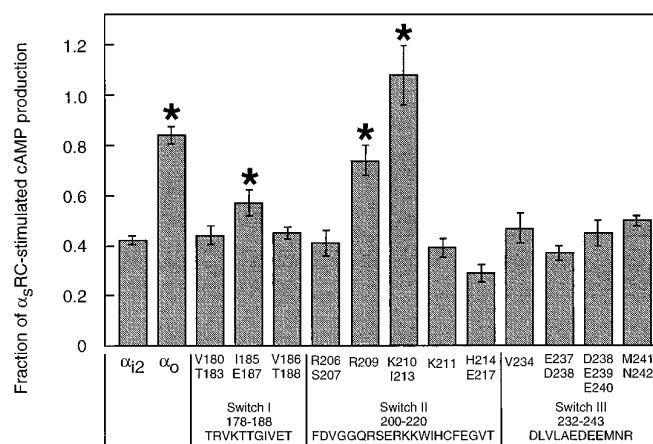


FIG. 1. Alanine substitutions of solvent-exposed residues in Switches I-III. The residues that were substituted by alanines in each construct and the residue ranges and sequences of Switches I-III in α_{i2} are indicated. All constructs include the GTPase-inhibiting arginine to cysteine mutation (R179C in α_{i2} and α_o) and the EE epitope. cAMP accumulation in 10^6 HEK-293 cells transfected with 0.1 μ g of vector containing α_s RC and 0.3 μ g of vector containing the indicated α subunit constructs is shown. The amount of cAMP accumulation in cells transfected with α_s RC alone is set at 1.0, and the values from cells co-transfected with the indicated constructs are expressed relative to this value. Asterisks indicate cAMP values of constructs with significantly decreased abilities to inhibit cAMP accumulation ($p < 0.05$) compared with α_{i2} RCEE. cAMP levels in [3 H]adenine-labeled cells were determined as described under "Experimental Procedures." Each value represents the mean \pm S.E. of at least three independent experiments.

treated trypsin (Sigma T-8642) was added to a final concentration of 5 μ g/ml, and the mixture was incubated for 5 min at 30 °C. The digestion was terminated by adding soybean trypsin inhibitor to a final concentration of 1 mg/ml. The samples were then resolved by SDS-polyacrylamide electrophoresis (10%), transferred to nitrocellulose, and probed with the anti-EE monoclonal antibody (22), which was purified from hybridoma supernatants using E-Z-SEP reagents (Middlesex Sciences, Inc). The antigen-antibody complexes were detected using an anti-mouse horseradish peroxidase-linked antibody according to the ECL Western blotting protocol (Amersham Life Science, Inc.).

RESULTS

Characterization of Mutant α_{i2} Constructs Using cAMP Assay—To characterize mutant α_{i2} subunits after transient expression in HEK-293 cells, two features were included, as in a previous study (14), to enable measurement of their functions without interference from the activities of the α_i proteins endogenous to these cells. First, a conserved arginine (R179C) was replaced by cysteine. This mutation constitutively activates α_{i2} by inhibiting its GTPase activity (20) and made it possible to measure inhibition of adenylyl cyclase without requiring receptor-mediated activation of the mutant α_{i2} subunits. Second, the α_{i2} constructs include an epitope from an internal region of polyoma virus medium T antigen, referred to as the EE epitope (22), which does not interfere with the α_{i2} -adenylyl cyclase interaction (27).

We measured the ability of recombinant α subunits to inhibit adenylyl cyclase in HEK-293 cells by co-expressing them with the constitutively activated α_s mutant, α_s RC, in which arginine 201 is mutated to cysteine (21). As in a previous study (14), transfection with 0.1 μ g of vector containing α_s RC resulted in an approximately 18-fold increase in cAMP production compared with cells transfected with vector alone. Co-transfection with 0.3 μ g of vector containing α_{i2} RCEE resulted in ~60% inhibition of the cAMP response to α_s RC, while co-transfection with the same amount of vector containing α_o RCEE inhibited the response to α_s RC by only ~15% (Fig. 1). We used α_o RCEE as a negative control because α_o has been shown to have little or no ability to inhibit adenylyl cyclase (10, 11).

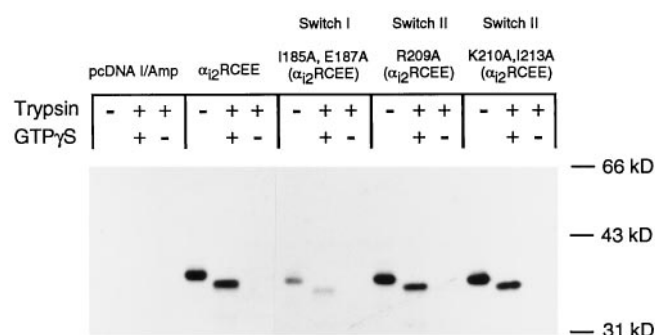


FIG. 2. **Expression and trypsin sensitivity of α_{12} constructs containing mutations in Switches I and II.** 12.5×10^6 HEK-293 cells were transfected with $2 \mu\text{g}/10^6$ cells of vector alone or vector containing the indicated α_{12} constructs, and membranes were prepared, treated with trypsin, and immunoblotted as described under "Experimental Procedures." The first lane in each set is the control (no trypsin). The second and third lanes show the result of trypsin digestion in the presence or absence, respectively, of $\text{GTP}\gamma\text{S}$.

Alanine Substitutions within Conformational Switch Regions—Since the GTP-bound form of α_{12} inhibits adenylyl cyclase much more effectively than the GDP-bound form does (11), it was surprising that the effector-specifying region of α_{12} , as defined by the 78-residue segment, residues 245–322 (14), did not include any of the three regions, Switches I–III (6, 8), that undergo GTP-dependent conformational changes. However, the sequences of these regions are highly conserved in α_{12} and α_q . 7 of the 11 Switch I residues, 18 of the 21 Switch II residues, and 6 of the 12 Switch III residues are identical in the sequences of α_{12} and α_q . Therefore, the importance of these regions as effector binding sites could have been missed using homologous sequence substitutions.

To directly test the importance of Switches I–III as effector contact sites, we mutated solvent-exposed residues within each of these regions to alanine residues. Substitutions using alanine residues eliminate the side chain beyond the β carbon but generally do not alter the main chain conformation and do not impose significant electrostatic or steric effects (28). We identified clusters of solvent-exposed residues by inspection of the x-ray crystal structures of the $\text{GTP}\gamma\text{S}$ -bound forms of α_{11} (7) and α_t (5) and calculations of fractional accessibility values (29) from the coordinates. As shown in Fig. 1, we mutated three clusters of residues in Switch I (6 residues), five clusters of residues in Switch II (8 residues), and four clusters of residues in Switch III (7 residues).

We found that alanine substitutions of three residues in Switch II, Arg-209, Lys-210, and Ile-213, blocked $\alpha_{12}\text{RCEE}$ from inhibiting adenylyl cyclase (Fig. 1). These residues are located in the middle of the α_2 helix and are highly conserved among α subunits (see Fig. 7). We previously found that substituting α_{12} homologs for three α_s Switch II residues located at the carboxyl terminus of α_2 and in the α_2/β_4 loop, Gln-236, Asn-239, and Asp-240 (see Fig. 7), specifically prevents α_s from activating adenylyl cyclase (12). Although not conserved between α_s and α_{12} , these residues are identical in the sequences of α_{12} and α_q and therefore were not tested in our previous α_{12}/α_q chimera studies (14). The α_t and α_{11} homologs of the first two of these residues are solvent-exposed in the structures. Alanine substitutions of the corresponding α_{12} residues, His-214 and Glu-217, did not block the ability of α_{12} to inhibit adenylyl cyclase (Fig. 1).

We did not obtain evidence that either the Switch I or Switch III regions of α_{12} are specifically involved in inhibition of adenylyl cyclase (Fig. 1). The only substitutions that caused a partial loss of function were in residues Ile-185 and Glu-187 in Switch I (Fig. 1). However, these substitutions also greatly

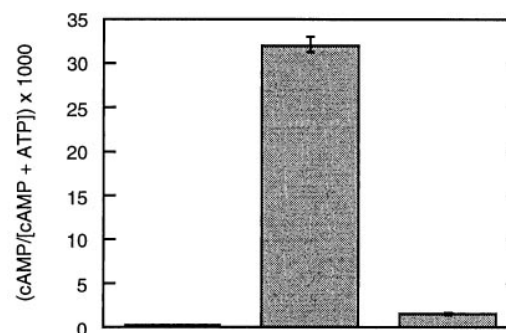


FIG. 3. **A conserved region of Switch II is specifically required for the activation of adenylyl cyclase by α_s .** Top part of figure shows cAMP accumulation in 10^6 HEK-293 cells transfected with $1.5 \mu\text{g}$ of vector containing $\alpha_s\text{RCEE}$ or (R232A,I235A) $\alpha_s\text{RCEE}$ or with vector alone. cAMP levels in [^3H]adenine-labeled cells were determined as described under "Experimental Procedures." Conversion of ATP to cAMP is expressed as $[\text{cAMP}]/([\text{cAMP}] + [\text{ATP}]) \times 1000$ (44). Each value represents the mean \pm S.E. of three independent experiments.

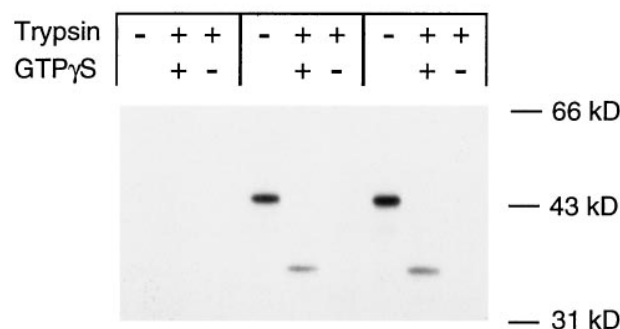


FIG. 3. **A conserved region of Switch II is specifically required for the activation of adenylyl cyclase by α_s .** Top part of figure shows cAMP accumulation in 10^6 HEK-293 cells transfected with $1.5 \mu\text{g}$ of vector containing $\alpha_s\text{RCEE}$ or (R232A,I235A) $\alpha_s\text{RCEE}$ or with vector alone. cAMP levels in [^3H]adenine-labeled cells were determined as described under "Experimental Procedures." Conversion of ATP to cAMP is expressed as $[\text{cAMP}]/([\text{cAMP}] + [\text{ATP}]) \times 1000$ (44). Each value represents the mean \pm S.E. of three independent experiments. Bottom part of figure shows expression and trypsin sensitivity of these constructs. 12.5×10^6 HEK-293 cells were transfected with $6 \mu\text{g}/10^6$ cells of vector alone or vector containing $\alpha_s\text{RCEE}$ or (R232A,I235A) $\alpha_s\text{RCEE}$, and membranes were prepared, treated with trypsin, and immunoblotted as described under "Experimental Procedures." The first lane in each set is the control (no trypsin). The second and third lanes show the result of trypsin digestion in the presence or absence, respectively, of $\text{GTP}\gamma\text{S}$.

reduced the expression level of $\alpha_{12}\text{RCEE}$ (see below).

Criteria for Specificity of Mutations—Mutations that prevent $\alpha_{12}\text{RCEE}$ from inhibiting adenylyl cyclase could do so for reasons other than disruption of residues that interact with this effector. Therefore, we subjected constructs with these mutations to the following criteria for specificity. The first criterion was that the mutants should be expressed at wild-type levels in HEK-293 cell membranes. This criterion was tested by performing immunoblots on membranes prepared from cells expressing the mutants. The second criterion was that the mutants should be able to bind GTP and undergo the GTP-dependent conformational change that is detected as the acquisition of resistance to trypsin cleavage (30–32). This second criterion is quite stringent because it requires not only proper GTP binding but also the ability to respond to this binding with an activating conformational change. Under the conditions of this assay, in the presence of $\text{GTP}\gamma\text{S}$, trypsin removes a short segment from the amino terminus but leaves most of the protein intact (Fig. 2). However, in the absence of $\text{GTP}\gamma\text{S}$, trypsin degrades $\alpha_{12}\text{RCEE}$ to small fragments not seen on SDS-polyacrylamide gels.

We found that the Switch II mutant constructs, (R209A) $\alpha_{12}\text{RCEE}$ and (K210A,I213A) $\alpha_{12}\text{RCEE}$, were expressed as well as $\alpha_{12}\text{RCEE}$ and achieved the GTP-dependent activated conformation, as measured by the trypsin assay (Fig. 2). Therefore, by our

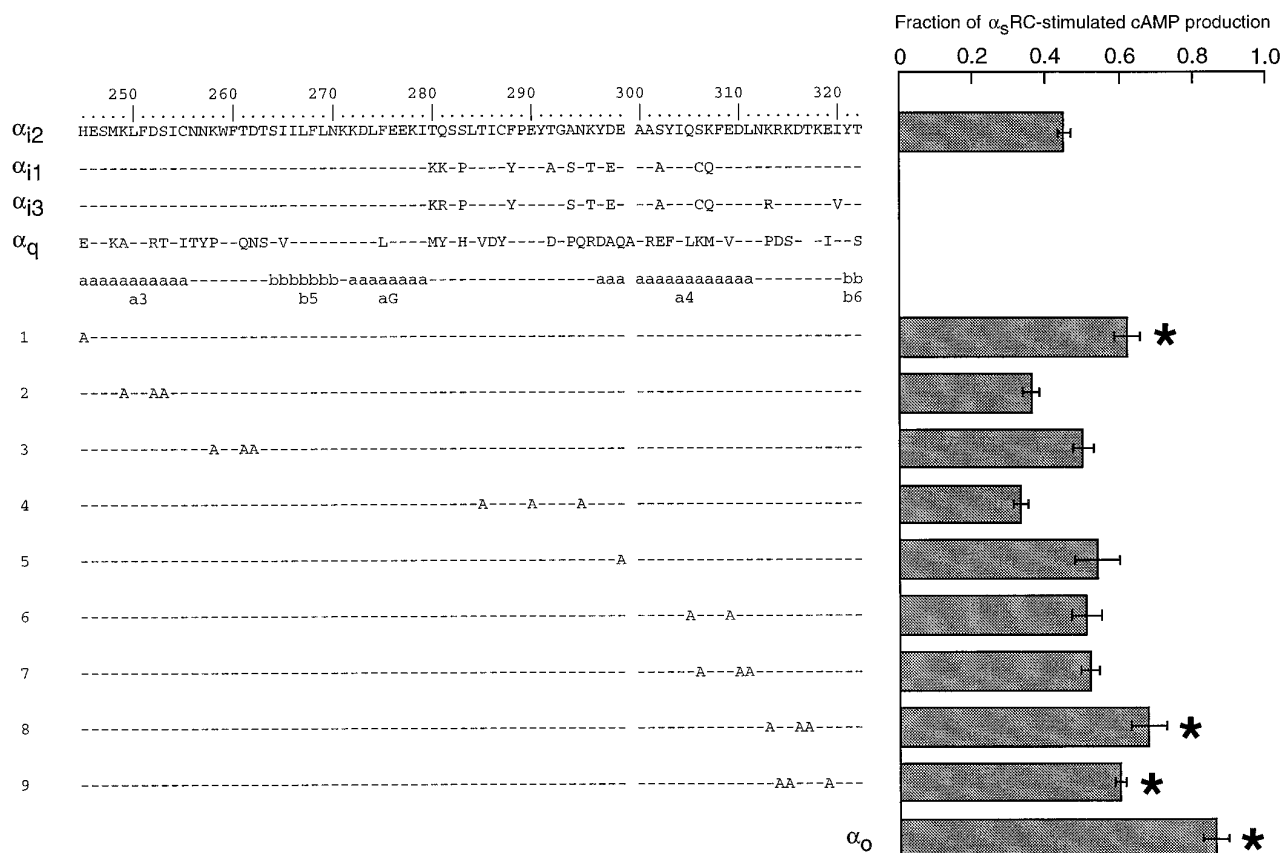
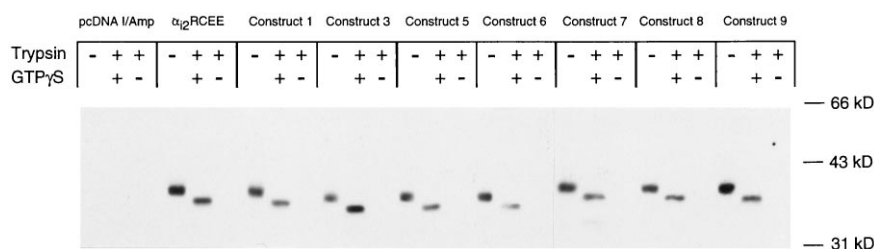


FIG. 4. Alanine substitutions of solvent-exposed residues within the 78-residue α_{i2} segment. The top sequence is that of α_{i2} residues 245–322. Below that are the sequences of α_{i1} , α_{i3} , and α_q , with residues identical to α_{i2} residues represented by dashes. The numbered sequences represent individual mutant constructs with alanine substitutions at the indicated positions. All constructs include the GTPase-inhibiting arginine to cysteine mutation (R179C in α_{i2} and α_o) and the EE epitope. Shown next to each construct is the cAMP accumulation in 10^6 HEK-293 cells transfected with 0.1 μ g of vector containing α_s RC and 0.3 μ g of vector containing the indicated α subunit construct. The amount of cAMP accumulation in cells transfected with α_s RC alone is set at 1.0, and the values from cells co-transfected with the indicated constructs are expressed relative to this value. Asterisks indicate cAMP values of constructs with significantly decreased abilities to inhibit cAMP accumulation ($p < 0.05$) compared with α_{i2} RCEE. cAMP levels in [3 H]adenine-labeled cells were determined as described under “Experimental Procedures.” Each value represents the mean \pm S.E. of at least three independent experiments.

FIG. 5. Expression and trypsin sensitivity of α_{i2} constructs containing mutations within the 78-residue α_{i2} segment. 12.5×10^6 HEK-293 cells were transfected with 2 μ g/ 10^6 cells of vector alone or vector containing the indicated α_{i2} constructs, and membranes were prepared, treated with trypsin, and immunoblotted as described under “Experimental Procedures.” The first lane in each set is the control (no trypsin). The second and third lanes show the result of trypsin digestion in the presence or absence, respectively, of GTP γ S.



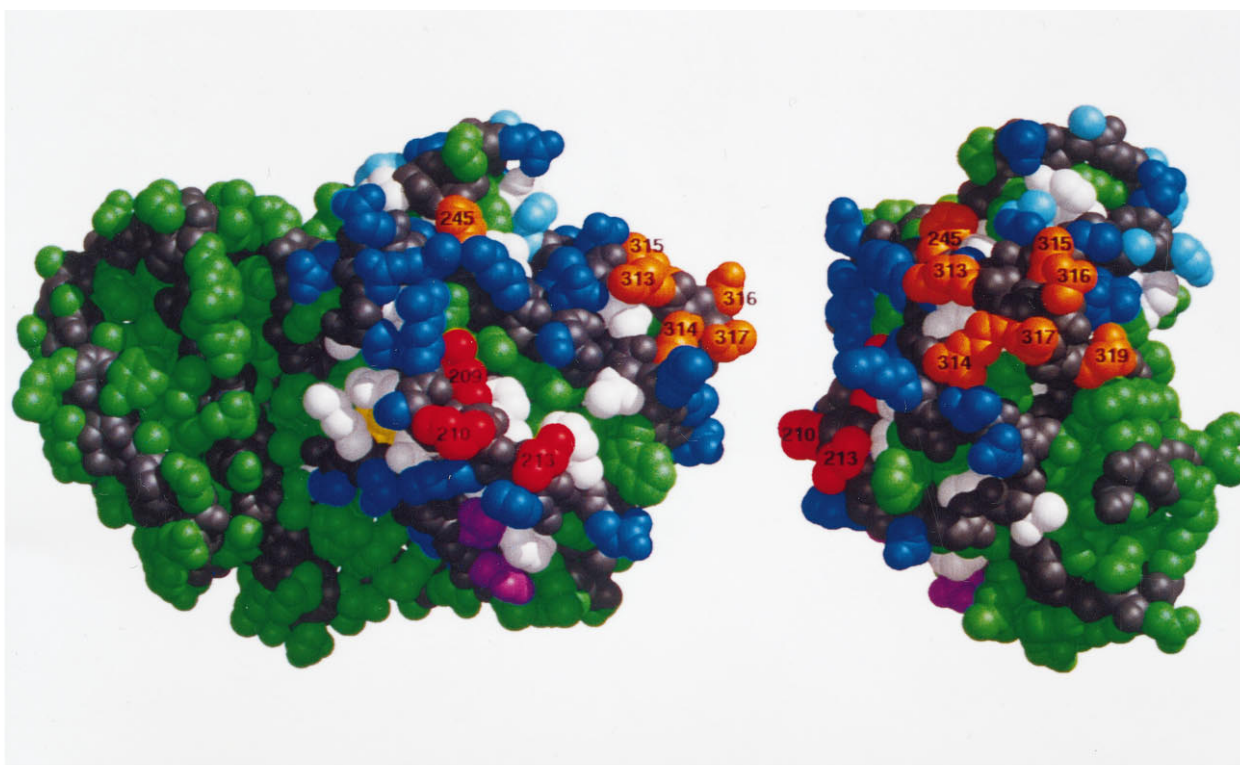
criteria, residues Arg-209, Lys-210, and Ile-213 are specifically required for interaction with adenyl cyclase. In contrast, although the Switch I mutant construct, (I185A,E187A) α_{i2} -RCEE, exhibited resistance to trypsin in the presence of GTP γ S, it was expressed very poorly (Fig. 2). The role of residues Ile-185 and Glu-187 in effector interaction is, therefore, uncertain.

In the course of these studies, we mutated the α_{i2} residue, Arg-209, that corresponds to the GTP γ S-protected trypsin site determined by amino-terminal sequencing of tryptic peptides from α_i and α_o (31). Elimination of this cleavage site would be expected to result in an α subunit that was resistant to trypsin cleavage in both the presence and absence of GTP γ S. However, (R209A) α_{i2} RCEE was resistant to trypsin cleavage in the presence but not the absence of GTP γ S (Fig. 2). Similar results

were obtained upon mutation of each of the other potential trypsin sites in Switch II, Arg-206,² Lys-210 (Fig. 2), and Lys-211,² as well as mutation of all four residues simultaneously.² These results suggest that, although Switch II may contain cleavage sites that change conformation upon GTP binding, there are also other sites outside of this region that are preferentially cleaved by trypsin in the absence compared with the presence of GTP γ S. Nevertheless, the ability of the trypsin assay to detect GTP-dependent conformational changes in Switch II is demonstrated by the fact that the Switch II α_s mutant, G226A α_s , which is unable to undergo the activating conformational change required for dissociation from $\beta\gamma$,

² C. H. Berlot, unpublished observations.

A



B

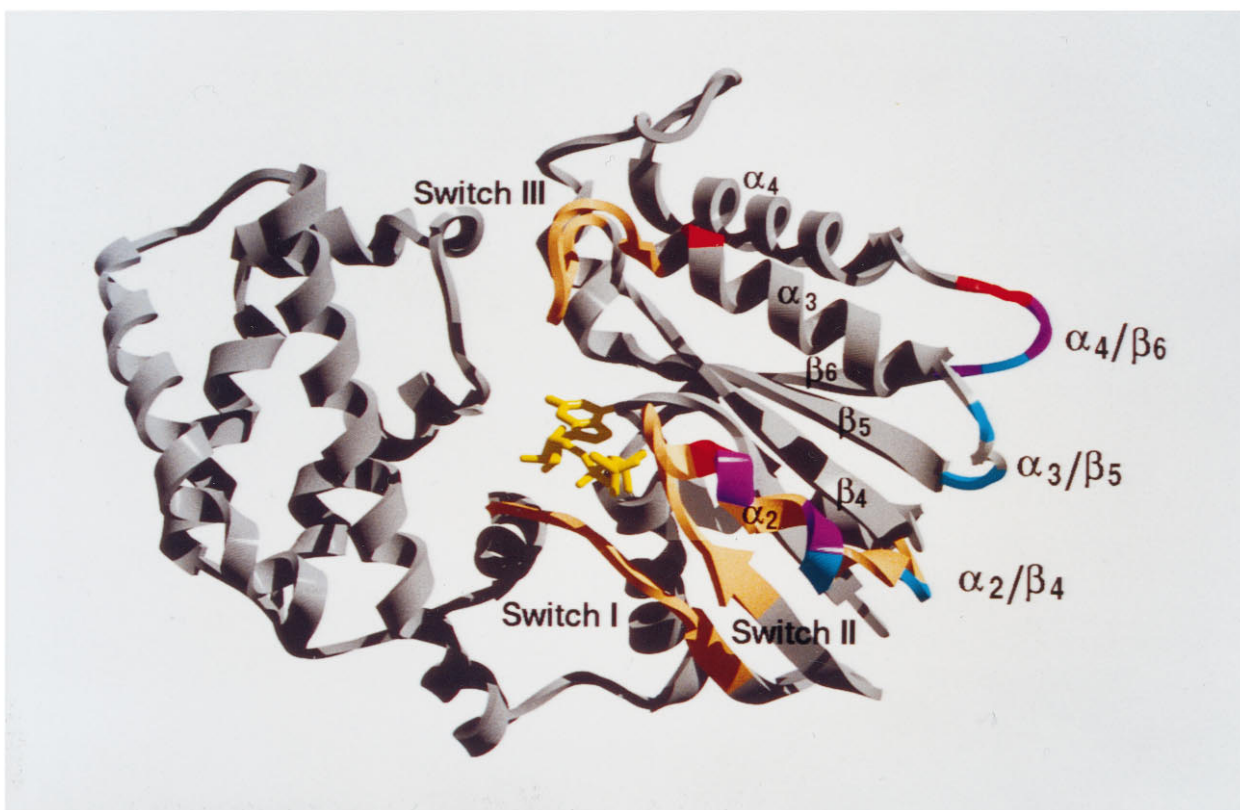


FIG. 6. Mapping of effector-interacting residues of α_{i2} and α_s onto the x-ray crystal structure of the GTP γ S-bound form of α_{i1} . A, space-filling model showing α_{i2} residues required for inhibition of adenylyl cyclase. Residues that were mutated are shown in red, magenta, orange, and dark blue, as follows. Residues in Switch II specifically required for inhibition of adenylyl cyclase are red. Residues in Switch I in which mutations reduce both inhibition of adenylyl cyclase and expression level are magenta. Residues within the 78-residue segment in which mutations cause a partial loss of adenylyl cyclase inhibition are orange. Residues in which mutations do not affect inhibition of adenylyl cyclase are dark blue. Residues that were not mutated in this study are shown in green, light blue, and white, as follows. Residues outside of the 78-residue segment and Switches I-III are green. Also green are the residues within the 78-residue α_{i2} segment that are conserved between α_{i2} and α_{i3} . The α_{i2} residues within this segment that differ from α_{i3} residues but are not identical among the three α_i isoforms are light blue. Residues in Switches I-III that were not mutated and residues within the 78-residue segment that differ from α_{i3} residues and are conserved among the three α_i isoforms, but were not mutated, are white. Main chain backbone atoms are gray. The GTP is yellow. The numbers on the model refer to α_{i2} residues. The model on the right is rotated approximately 90° about the vertical axis relative to the model on the left. B, ribbon diagram showing comparison of

does not acquire trypsin resistance in the presence of GTP γ S (32, 33).

A Conserved Region of Switch II Is Specifically Required for the Effector Interactions of Both α_s and α_{i2} —To determine whether the highly conserved middle region of Switch II (see Fig. 7) is required for the activation of adenylyl cyclase by α_s , we tested the effects of substituting alanines for the α_s residues (Arg-232 and Ile-235) that correspond to Lys-210 and Ile-213 in α_{i2} . We introduced these substitutions into α_s RCEE, which contains the EE epitope, previously shown to have no effect on the interaction between α_s and adenylyl cyclase (34). The substitutions almost entirely prevented α_s RCEE from activating adenylyl cyclase without affecting the GTP-dependent conformational change measured by the trypsin assay (Fig. 3). Thus, the same region of Switch II is required for the interaction of both α_s and α_{i2} with adenylyl cyclase.

Alanine Substitutions within the 78-Residue Segment—Since α_{i2} , but not α_q , inhibits adenylyl cyclase (10, 13) and an $\alpha_q/\alpha_{i2}/\alpha_q$ chimera containing only 78 α_{i2} residues (245–322) inhibits adenylyl cyclase as well as α_{i2} does (14), the α_{i2} residues that specify inhibition of adenylyl cyclase must be located within this 78-residue segment. To identify these effector-specifying residues, we tested the effects of mutating nine clusters of solvent-exposed residues (22 residues total) to alanine residues (Fig. 4). Within the 78-residue segment of α_{i2} , 65 residues are identical among the three α_i isoforms, which have equal abilities to inhibit adenylyl cyclase (11). Of these 65 residues, 28 are different in α_q and therefore might account for the ability of α_q , but not α_i , to inhibit adenylyl cyclase. 20 of the substitutions were in residues that are identical among the three α_i subunits, and 18 were in residues that differ between α_{i2} and α_q . The thoroughness of our mutational analysis is illustrated in Fig. 6A.

As shown in Fig. 4, substitutions of three sets of residues: His-245 (Construct 1), Lys-313, Asp-316, and Thr-317 (Construct 8), and Arg-314, Lys-315, and Glu-319 (Construct 9), significantly reduced inhibition of adenylyl cyclase. However, in contrast to the Switch II mutations, which entirely blocked the ability of α_{i2} RCEE to inhibit adenylyl cyclase, the mutations in Constructs 1, 8, and 9 had only partial effects. The other six clusters of mutations (15 residues) did not significantly impair the ability of α_{i2} RCEE to inhibit adenylyl cyclase.

All of the constructs that inhibited adenylyl cyclase to a similar or decreased extent compared with α_{i2} RCEE were expressed in HEK-293 cell membranes and were able to undergo the GTP-dependent conformational change that results in increased resistance to trypsin digestion (Fig. 5). However, since scanning densitometry of immunoblots showed that Constructs 1, 8, and 9 were expressed at lower levels than α_{i2} RCEE was, their decreased abilities to inhibit adenylyl cyclase may be due to effects of the mutations on protein folding and/or stability. Nevertheless, since we have substituted alanines for the majority of solvent-exposed residues within the effector-specifying 78-residue segment (see Fig. 6A) and the other substitutions did not significantly reduce adenylyl cyclase inhibition, the residues in Constructs 1, 8, and 9 are, by default, the most likely candidates for specifying inhibition of adenylyl cyclase.

Comparison of the Effector-Interacting Surfaces of α_{i2} and α_s —We used the x-ray crystal structure of the GTP γ S-bound form of α_{i1} (7) to map the results of our mutagenesis studies. 88% of the residues in α_{i2} can be aligned with identical residues

Switch II

α_{i2} (200–221) F D V G G Q R S E R K K W T H C F E G V T A
 α_s (222–243) F D V G G Q R D E R R K W I Q C F N D V T A
 α_t (195–216) F D V G G Q R S C R K K W I H C F E G V T C
 b b b - - - a a a a a a a a - - - - - b
 β_3 α_2 β_4

α_4/β_6 Loop

α_{i2} (300–321) A A S Y I Q S K F E D L N K R K D T K E I Y
 α_s (337–358) A K Y F I R D E F L R I S T A S G D G R H Y
 α_t (295–316) A G N Y I K V Q F L E L N M R R D V K E I Y
 a a a a a a a a a a - - - - - b
 α_4 β_6

FIG. 7. Comparison of effector-interacting residues of α_{i2} , α_s , and α_t in Switch II and in the α_4/β_6 loop. Residue numbers of α_{i2} , α_s , and α_t in the Switch II and α_4/β_6 regions are indicated in parentheses. Mutations of boxed residues impaired effector interaction. Mutations of underlined residues did not impair effector interaction. Mutation of the circled glutamate residue in Switch II of α_t caused constitutive activation of PDE. Data for α_{i2} are from Figs. 1 and 4. Data for α_s are from Fig. 3 and Berlot and Bourne (12). Data for α_t are from Spickofsky *et al.* (17), Faurobert *et al.* (36), and Mittal *et al.* (37).

in α_{i1} , while 67% of the α_{i1} residues can be aligned with identical residues in α_t . Since the structures of the active (GTP γ S-bound) forms of α_{i1} (7) and α_t (5) are virtually identical, the structure of α_{i1} is an excellent model for that of α_{i2} . Our mutagenesis analysis of Switches I–III in α_{i2} and the 78-residue effector-specifying α_{i2} segment, residues 245–322, focused on solvent-exposed residues. In addition, most of the alanine substitutions in the 78-residue segment were of residues that are: 1) different from the homologous α_q residues and 2) conserved among the α_i isoforms. The thoroughness of this study is demonstrated by the fact that the residues in Switches I–III that were not mutated and the residues in the 78-residue segment that meet criteria 1 and 2 but were not mutated represent a very small fraction of the available surface area (shown in white in Fig. 6A).

The alanine substitutions that caused the largest decrease in the ability of α_{i2} RCEE to inhibit adenylyl cyclase were in the middle of the α_2 helix in Switch II (red in Fig. 6A). The effector-interacting surfaces of α_s and α_{i2} overlap exactly in this region (magenta in Fig. 6B) where the sequences of the two α subunits are highly conserved (Fig. 7). However, the α_2/β_4 loop at the carboxyl-terminal end of Switch II is important for the interaction of α_s (12) but not α_{i2} (Fig. 1) with adenylyl cyclase (blue in Fig. 6B).

The alanine substitutions within the 78-residue effector-specifying segment that caused a moderate reduction in the ability of α_{i2} RCEE to inhibit adenylyl cyclase (orange in Fig. 6A) were in the amino terminus of α_3 (Construct 1) and in the α_4/β_6 loop (Constructs 8 and 9) (Fig. 6B). The amino terminus of α_3 (red in Fig. 6B) is important for the effector interactions of α_{i2} (Fig. 4), but not α_s (12), while mutations in the α_3/β_5 loop (blue in Fig. 6B) disrupt interaction between α_s and adenylyl cyclase (12) but do not have a significant effect on the α_{i2} -adenylyl cyclase interaction (Fig. 4). Residues in the α_4/β_6 loop found to be important for specifying the effector interactions of both α_{i2} and α_s are magenta in Fig. 6B.

DISCUSSION

The studies reported here investigated two key aspects of α subunit-effector interactions, GTP-dependence and specificity. We found that in the case of α_{i2} , these two components of effector interaction are mediated by distinct regions of surface residues. GTP-dependent effector interaction is mediated by Switch II residues that are conserved among α subunits (Fig. 1) while specificity (inhibition of adenylyl cyclase) is mediated by nonconserved residues (the amino terminus of α_3 and the α_4/β_6 loop) outside of the conformational switch regions (Fig. 4). In contrast, in the case of α_s , Switch II plays a role in regulating both the GTP dependence of effector interaction as well as effector specificity. The conserved Switch II region is required for GTP-dependent activation of adenylyl cyclase (Fig. 3) while nonconserved Switch II residues, as well as residues outside of the conformational switch regions (the α_3/β_5 and α_4/β_6 loops), are involved in regulating effector specificity (12). In the case of α_t , the conformational switch regions and regions that don't switch conformation (α_3 and the α_3/β_5 loop) interact with distinct regions of the effector molecule, PDE (35).

Taken together, our results and those of others indicate that two α subunit regions, Switch II and the α_4/β_6 loop, may be important for effector interactions in general (Fig. 7). The conserved middle region of Switch II has been shown to be important for the interaction between α_t and PDE. Mutation of a conserved tryptophan in α_t reduces binding to PDE (36) while mutation of a conserved glutamate causes constitutive activation of PDE by the GDP-bound form of α_t (37). The α_4/β_6 loop is involved in specifying the effector interactions of at least three α subunits (Fig. 7). We previously found that replacement of α_s residues in this region by their α_{i2} homologs prevents α_s from activating adenylyl cyclase without preventing the mutant protein from attaining the GTP-dependent active conformation (12). Rarick *et al.* (16) found that a 22-amino acid peptide (α_t residues 293–314) activates PDE. Within this region, Spickofsky *et al.* (17) identified five residues in which substitutions of homologs from other α subunits block PDE activation by peptides. Three of these residues are in the α_4 helix and two are in the α_4/β_6 loop. Mutations in the α_4/β_6 loop of α_s and α_{i2} , but not in α_4 cause decreases in effector modulation. In the case of α_q , α_4 and the α_4/β_6 loop have been implicated in PLC activation in studies using peptides (38). However, chimera studies showed this region could be replaced with α_s sequence without affecting PLC activation (39).

Since α_s and α_{i2} have opposite effects on adenylyl cyclase activity, the conserved region of Switch II required for the effector interactions of both α subunits is most likely involved in regulating GTP-dependent effector binding. Of the three residues found to be important for inhibition of adenylyl cyclase by α_{i2} , Arg-209 and Ile-213 are identical in the sequences of α_s and α_{i2} (see Fig. 7). The third residue is conserved but not identical between the two α subunits (Lys-210 in α_{i2} , Arg-232 in α_s). However, α_{i2}/α_s chimera studies showed that substitution of lysine for arginine at position 232 in α_s has no effect on activation of adenylyl cyclase (12). Furthermore, the α_q residue corresponding to Lys-210 is an arginine residue and α_q/α_{i2} chimera studies showed that substitution of arginine at this position does not affect inhibition of adenylyl cyclase (14). Therefore, these Switch II residues do not determine the nature of adenylyl cyclase modulation by α_s and α_{i2} .

Although all α subunits are conserved in this Switch II region, other α subunits do not modulate adenylyl cyclase, with the exception of a weak inhibition of type I adenylyl cyclase by α_o (11). A possible explanation for this selectivity is that other α subunits contain residues that preclude a productive adenylyl cyclase interaction. If so, then replacing α_{i2} residues in the

amino terminus of α_3 and in the α_4/β_6 loop with the homologous residues from α_q or other α subunits might cause a larger reduction in ability to inhibit adenylyl cyclase than was observed for alanine substitutions.

Our studies show that the effector-specifying regions of α_s and α_{i2} overlap but are not identical (see Fig. 6B). Studies using α subunit chimeras localized the region of α_{i2} that specifies inhibition of adenylyl cyclase to a 78-residue segment (amino acids 245–322) that extends from α_3 to β_6 (14). Residues corresponding to two of the three α_s regions that specify activation of adenylyl cyclase (12, 15), the α_3/β_5 and α_4/β_6 loops, are included in this segment. The only region of overlap that we have found among the effector-specifying regions of α_s and α_{i2} is in the α_4/β_6 loop. Effector-specifying regions unique for α_s are located in the α_3/β_5 loop and in the carboxyl-terminal part of Switch II (12). Similarly, mutation of a single residue in the amino terminus of α_3 reduces the ability of α_{i2} to inhibit adenylyl cyclase but is not required for the activation of adenylyl cyclase by α_s (12).

Since both α_s and α_{i2} interact with adenylyl cyclase, the effector-specifying residues of each α subunit presumably determine whether activation or inhibition will result from α subunit binding. However, the effector-specifying residues of α_s appear to contribute more to the interaction with adenylyl cyclase than do those of α_{i2} . Substitutions in the effector-specifying segment of α_{i2} do not cause as large of a decrease in the ability to inhibit adenylyl cyclase as do substitutions in the conserved middle part of Switch II. However, mutations in two of the effector-specifying regions of α_s , the nonconserved carboxyl-terminal part of Switch II and the α_3/β_5 loop, decrease effector activation to the same extent as do mutations in the conserved Switch II region.² Consistent with our results, Tausig *et al.* (11) found that replacing α_{i1} residues with α_s homologs in the α_3/β_5 loop results in an α subunit that weakly activates certain adenylyl cyclase isoforms. Thus, the effector-specifying regions of α_s appear to be dominant over those of α_{i2} .

Mutagenesis studies of hGH and its receptor, for which a structure of the hormone-receptor complex is available (40), have characterized the functional importance of residues in the binding interface. Individual replacements of residues in hGH (41) and its receptor (42) demonstrated that only a small subset of the residues at the center of the contact region contribute substantially to binding affinity. However, hGH residues in the periphery of the interface, which do not contribute much to the affinity of binding (41), are important for the specificity of binding (43).

In a similar manner, our studies of the interaction between α_{i2} and adenylyl cyclase implicate Switch II residues as being the major contributors to this binding interaction. Substitutions in the effector-specifying segment of α_{i2} have a more modest effect on the ability of α_{i2} RCEE to inhibit adenylyl cyclase. In the absence of any structures of α subunit-effector complexes, we predict that interactions between these proteins will include the conserved Switch II region as well as nonconserved specificity regions but that, as seen in the case of hGH and its receptor (41, 42), the contact surfaces may be larger than the "functional epitopes" defined by our mutagenesis studies.

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